IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	**RICH, Ivan N.**
Serial No.	:	**10/059,521**
Filing Date	:	**January 29, 2002**
For	:	**High-Throughput Stem cell Assay of Hematopoietic Stem and Progenitor Cell Proliferation**
Examiner	:	**Gailene R. Gabel**
Art Unit	:	**5794**

EXPRESS MAIL

Mailing Label Number: EV 196815974 US

Date of Deposit: May 6, 2005

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DECLARATION UNDER 37 C.F.R. 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Dr. Ivan N. Rich, declare and state that:

1. I make this declaration in connection with the above-referenced U.S. patent application Serial No. 10/059,521. I am the inventor of this application and am familiar with it and its prosecution history, particularly the Office Action mailed on February 09, 2005. It is contended by the Office Action that elements from the hemoglobin-based cell proliferation based system of Bell et al. (US 2002/0120098 Al) may be combined with those from the luciferase-based assay of Crouch et al., ((1993) J. Immunol. Meth. 160: 81-88) to provide the functional assay method of the present invention, and as claimed in the above-referenced application.

2. Attached is my *Curriculum vitae* (Appendix A). In view of my education, training and experience, I respectfully submit that I consider myself, and am considered by my peers, to be an expert in the field to which this application pertains.

3. The following experiments were performed under my direction, supervision or control in the ordinary course of business. I respectfully submit that I am qualified to address the issues stated herein, and to introduce the experiments stated herein.

4. **Suppression by hemoglobin of detectable ATP-luciferase-generated luminescence.**

4(a). This experiment shows that if hemoglobin is present in the HALO luciferase-based assays of the proliferation of lympho-hematopoietic cell populations as taught and claimed in the above-referenced application, there is significant suppression of the luminescence generated by ATP-luciferase, thereby rendering the assays unable to correlate luminescence values to the proliferative status of the primitive hematopoietic cell populations.

4(b). The HALO assay, as claimed by the above-referenced application, comprises the steps of: (a) providing a cell population comprising primitive hematopoietic cells; (b) incubating the cell population in a cell growth medium comprising fetal bovine serum having a concentration of between 0% and 30% and methyl cellulose having a concentration of between about 0.4% and about 0.7%, and in an atmosphere having between about 3.5% oxygen and 7.5% oxygen; (c) contacting the cell population with a reagent capable of generating luminescence in the presence of ATP; and (d) detecting luminescence generated by the reagent contacting the cell population, the level of luminescence indicating the amount of ATP in the cell population, wherein the amount of ATP indicates the proliferative status of the primitive hematopoietic cells. The step of providing the primitive hematopoietic cells is disclosed in Example 1 of the application, wherein whole blood is subjected to a Ficoll gradient centrifugation to remove erythrocytes from the nucleated hematopoietic cells.

4(c). In this experiment, the suppression of luciferase-generated luminescence by hemoglobin, when included in the HALO assay as claimed in the above-referenced application, is shown. The proliferative potentials of various lympho-hematopoietic cell populations of human peripheral blood were assayed using the cytokine and luciferase-based HALO assay according to the methods as described in Examples 1 and 2 of, and claimed by, the above-referenced application. In this experiment, the lympho-hematopoietic cell populations were freed of erythrocytes by two different methods. One method, wherein the blood sample was

mixed with ammonium chloride to give an isotonic concentration of ammonium ions, resulted in the preferential lysis of red blood cells and the release of hemoglobin into the surrounding medium. Another method was density gradient centrifugation, according to the method as described in Example 2 of, and claimed by, the above-referenced application, which removed red cells and their hemoglobin contents.

4(d). In accordance with the method described in Example 2 of the above-referenced application, mononuclear cells (MNC) were prepared from human peripheral blood by density gradient centrifugation on Ficoll-Paque Plus by diluting the cell suspension 1:1 with sterile PBS and transferring up to 30 ml to a 15-20 ml cushion of Ficoll. Centrifugation was at 400 g for 20 minutes at room temperature. The supernatant was discarded and the cells were resuspended in 50 ml of sterile PBS and recentrifuged at 200 g for 10 minutes at room temperature. The supernatant was discarded and the cells were resuspended in IMDM to ensure a single cell suspension. A cell count was determined. Cells from peripheral blood mononucleocytes (MNCs) were prepared at a final concentration of 2×10^6 cells/ml.

4(e). After the lysis procedure and washing of the cells in cell growth medium, microscopical observation of the cultures showed erythrocyte ghosts and cell debris that is very difficult to remove. Lysis of the erythrocyte cell membranes released the hemoglobin contents of the erythrocytes into the surrounding medium. There was almost complete removal of the hemoglobin-bearing erythrocytes by Ficoll-based density gradient, which resulted in a suspension of hematopoietic cells significantly free of cell-bound or free hemoglobin.

4(f). In the HALO assay system according to Examples 1 and 2 of, and claimed by, the above-referenced application, the following components were mixed in sterile tubes in the following order, so that the final total volume of the culture mixture was 600 µl, or multiples thereof: cell cultures; methyl cellulose (stock at 2.6% v/v), 160 µl, (final concentration, 0.7% v/v); fetal bovine serum (FBS), 180 µl; α-thioglycerol, 6 µl at a final concentration of 1×10^{-4} M; human or bovine iron-saturated transferrin, 6 µl, final concentration of 1×10^{-10} g/ml; growth factors, individually or in combination, were selected from the following: erythropoietin, 1-3 U/ml; granulocyte-macrophage colony stimulating factor, 10-20 ng/ml; granulocyte colony stimulating factor, 10-20 ng/ml; macrophage colony stimulating factor, 10-20 ng/ml; thrombopoietin, 50 ng/ml; stem cell factor, 50 ng/ml; interleukin-1, 10-20 ng/ml; interleukin-2, 2-10 ng/ml; interleukin-3, 20 ng/ml; interleukin-6, 20 ng/ml; interleukin-7, 10 ng/ml. The

volume added depended upon the concentration of the cytokine/growth factor stock solution, but typically were not greater than 6-10 µl. All growth factors were diluted in IMDM containing either 5% FBS or 1% BSA; cells diluted in IMDM to the required final concentration as described above and added at 60 µl.; IMDM added to give a final stock solution volume of 600 µl (or multiples thereof).

4(g). Once the basic components were added, including the growth factors that were required for the specific cell type to be analyzed, the contents were vortexed to yield a homogenous mixture. The reaction mixes were left to stand for a few minutes before dispensing to the wells of a 96-well plate or other arrays of receptacles.

4(h). Aliquots of 100 µl each of a culture prepared as described above was withdrawn slowly and dispensed into each of the replicate wells of a 96-well plate while ensuring that little or none of the master mix touched the sides of the wells. Once the samples had been dispensed into the wells, the 96-well plate was placed in a fully humidified tissue culture incubator at 37° C. The cells were incubated in a low oxygen tension atmosphere of 5% CO_2 and 5% oxygen. The incubation period depended on the cell population to be tested.

4(i). After the incubation time elapsed, the reagents from a ViaLight HS™ kits (Lumitech) were prepared for use. ATP releasing reagent (100 µl aliquots) was added to wells of previously incubated 96-well plate. After dispensing the reagent, the contents of the wells were mixed at least 4-5 times. Once the ATP releasing reagent had been dispensed into all of the wells containing incubated cultures and mixed therein, the plates were incubated in the dark for 5 minutes. ATP monitoring reagent (20 µl) was then added to each of the well. The plates were immediately transferred to a plate reader and the luminescence measured using an integration time of 1000 ms. Measurements are given as relative luminescence units (RLU).

4(j). Two sets of assays were prepared according to the protocol described in paragraph 4(c) above. One set of assays incorporated lympho-hematopoietic cells prepared with ammonium chloride lysis of erythrocytes. The other set of assays, as claimed in the above-referenced application, incorporated the lympho-hematopoietic cells prepared by density gradient removal of the erythrocytes.

4(k). Fig. 1, Appendix B, shows the difference in the levels of ATP-luciferase-generated luminescence resulting from using normal human peripheral blood either lysed with ammonium chloride solution to destroy erythrocytes, or subjected to a Ficoll-Plaque Plus density

gradient centrifugation to remove erythrocytes, mature granulocytes and platelets as claimed in the above-referenced application.

4(l). Lysis of erythrocytes and the release of hemoglobin into the cell suspension used in the HALO assays claimed in the present application markedly suppresses the level of detectable luciferase-generated luminescence. The marked difference in suppression of the observed luminescence with lysed erythrocyte cell suspensions, compared to the results from cell suspensions of density gradient separated mononuclear cells, is primarily due to the hemoglobin released into the assay solution by erythrocyte lysis, which correlates with the formation of the cell membrane ghosts. Therefore, it is necessary to remove erythrocytes (and hence hemoglobin) from the primitive hematopoietic cell population, as described in Example 1 of the present application, before adding the cell population to the HALO luciferase-based assay system. This is contrary to any combination of Couch or Bell et al. as proposed by the Office Action, which will always include hemoglobin.

5. **Effect of intact erythrocytes on ATP-luciferase-generated luminescence**

5(a). This experiment shows that when intact erythrocytes are present in the HALO luciferase-based assays measuring the proliferation of lympho-hematopoietic cell populations, as claimed in the above-referenced application, there is either a false positive luminescence generated due to an increase in the oxygen content of the assay system, or a decrease of detectable luminescence due to absorbance by excess cells in the system. The presence of erythrocytes, therefore, prevents accurate measurements of lympho-hematopoietic cell proliferation by luciferase-generated luminescence, as taught and claimed by the above-referenced application.

5(b). In this experiment, total cell counts were performed on a sample of whole blood. A second count was then performed on the sample to which ZapOGlobin™ (Coulter Electronics, UK) had been added. ZapOGlobin is a reagent that specifically lyses red blood cells. The cell count after lysing the red blood cells is the remaining nucleated cell count. The difference between the two cell counts represents the erythrocyte concentration of the whole blood.

5(c). A luminescence dose response was then performed using the assay methods as recited in paragraph 4(e) above, and as taught in Examples 1 and 2 of, and claimed by, the above-referenced application. Luciferase-generated luminescence of increasing amounts of whole blood (comprising erythrocytes and nucleated cells) was measured. In this protocol, the

cells were titrated into a 96-well plate designed for reading the luminescence. Conditions were such that no cell proliferation occurred during the course of the assay. The cells were dispensed and the luminescence measured as described in paragraph 4(e) above.

5(d). Fig. 2, Appendix B, shows the effect on ATP-luciferase-generated luminescence of intact erythrocytes in the luciferase-based HALO assay system according to, and claimed by, the above-referenced patent application.

5(e). There was no difference between the luminescence as a function of the erythrocyte concentration and the luminescence as a function of the nucleated cell count; the responses paralleled each other. ATP-generated luminescence increased up to about 5×10^6 erythrocytes added per assay, or the equivalent of about 2.5×10^3 nucleated cells. Further increases in cell number resulted in the inhibition of luminescence.

5(f). The increase in the luminescence generated with whole blood in the HALO assay system claimed in above-referenced application is due to the oxygen contained in the erythrocytes as O_2-hemoglobin. This causes an imbalance in the ATP/luciferin/luciferase equilibrium reaction that, in the presence of oxygen, pushes the reaction in the direction of luminescence-generation. The subsequent decrease in luminescence at cells concentrations higher than about $2 \times 10^6/100\mu l$ is the result of cell density adsorbance.

6. The data presented herein shows that by including hemoglobin from lysed erythrocytes in the HALO assay system taught and claimed by the present application, ATP-luciferase-generated luminescence is suppressed. This result is in accordance with the data of Colin et al., ((2000) Gene Therapy 7: 1333-1336). Furthermore, intact erythrocytes added to the HALO assay system generate false high positive luminescence by increasing the oxygen content of the assay mixtures. Accordingly, any combination of the teachings in the Couch and Bell et al. documents cited in the Office Action mailed February 9, 2005 cannot provide the ATP/luciferin/luciferase-based assay of non-erythrocyte hematopoietic cell proliferation as claimed in the present application. The inclusion of free hemoglobin in such an assay system as taught by Bell et al. suppresses luminescence. Cell-bound hemoglobin generates false positive luminescence. Both effects will prevent the level of measured luminescence from providing an accurate indication of the amount of ATP in a lympho-hematopoietic cell population, wherein the amount of ATP indicates the proliferative status of the primitive hematopoietic cells, as taught and claimed by the present application. The data show the need to remove red blood cells from the primitive

hematopoietic cells of whole blood samples before performance of the luciferase-based assay of nucleated cell proliferation, as is taught in Example 1 of, and claimed by, the above-referenced application.

7. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 6/3/05

Ivan N. Rich

APPENDIX A
CURRICULUM VITAE OF IVAN N. RICH PH.D.

EDUCATION AND TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
University of Sussex, Falmer/Brighton,	BSc	1973	Biochemistry
University of Ulm, Ulm, Germany	PhD	1978	Human Biology
University of Chicago, Chicago, IL,	Post Doc	1981-1983	Hematopoiesis
University of Ulm, Ulm, Germany	Habilitation	1995	Exp. Hematology

POSITIONS AND HONORS.

6/2000 – Present	Chairman & CEO of HemoGenix, Inc.
4/1996 – 4/2000	Director of Basic Research, Division of Transplantation Medicine, Palmetto Richland Memorial Hospital, Columbia, South Carolina and Adjunct Professor of Medicine, Department of Immunology and Microbiology, University of South Carolina School of Medicine.
1/1983 – 4/1996	Associate Professor and Director of the Experimental Hematology Laboratory, Division of Transfusion Medicine of the University of Ulm in the German Red Cross Blood Bank, Ulm, Germany.
6/1981 – 12/1982	Postdoctoral Fellow, Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois
7/1973 – 6/1981	Research Associate, Division of Transfusion Medicine, Department of Internal Medicine III, University of Ulm, Ulm, Germany.

SELECTED PEER-REVIEWED PUBLICATIONS

1. **Rich** IN & Kubanek B. Erythroid colony formation (CFUe) in fetal liver and adult bone marrow and spleen from the mouse. Blut (1976), 33:171-180.
2. Opitz U, Seidel H-J & **Rich** IN. Erythroid stem cells in Rauscher virus infected mice. Blut. (1977), 35:35-44.
3. Hansi W, **Rich** IN, Heit W, Kubanek B & Heimpel H. Erythroid colony-forming cells in aplastic anemia. Brit. J. Haematol. (1977), 37:483-488.
4. **Rich** IN & Kubanek B. The ontogeny of erythropoiesis in the mouse detected by the erythroid colony-forming technique. I.Hepatic and maternal erythropoiesis. J. Embryol. exp. Morph. (1979), 50:57-74.
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16. **Rich** IN, Editor: Molecular and Cellular Aspects of Erythropoietin and Erythropoiesis. NATO Advanced Science Institute, Springer Verlag. (1987).
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RESEARCH SUPPORT.

1. SBIR Phase I entitled "An in vitro high throughput stem cell hemotoxicity assay" through the National Cancer Institute, 2001-2002 in response to the Program Announcement (PA-02-075), Innovative Toxicology Models. 2 R44 CA 93244-01
2. SBIR Phase II entitled "An in vitro high throughput stem cell hemotoxicity assay" through the National Cancer Institute, 2003-2005, in response to the Program Announcement (PA-02-075), Innovative Toxicology Models. 2 R44 CA 93244-02

APPENDIX B

Fig. 1

The Effect of Lysis and Ficoll-Separated (Density Gradient-Separated) Normal Human Peripheral Blood on the Growth Potential of Different Lympho-Hematopoietic Populations Measured using the HALO Platform

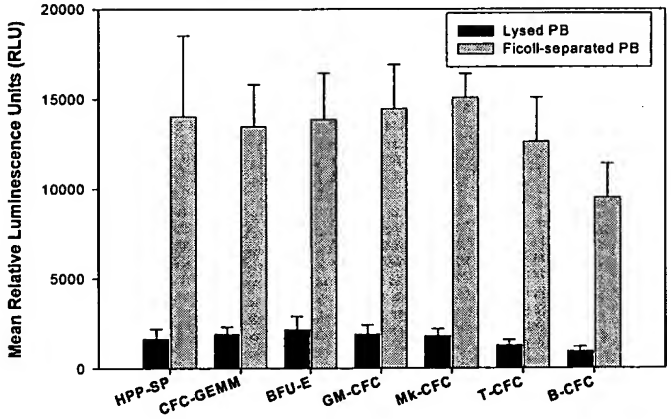
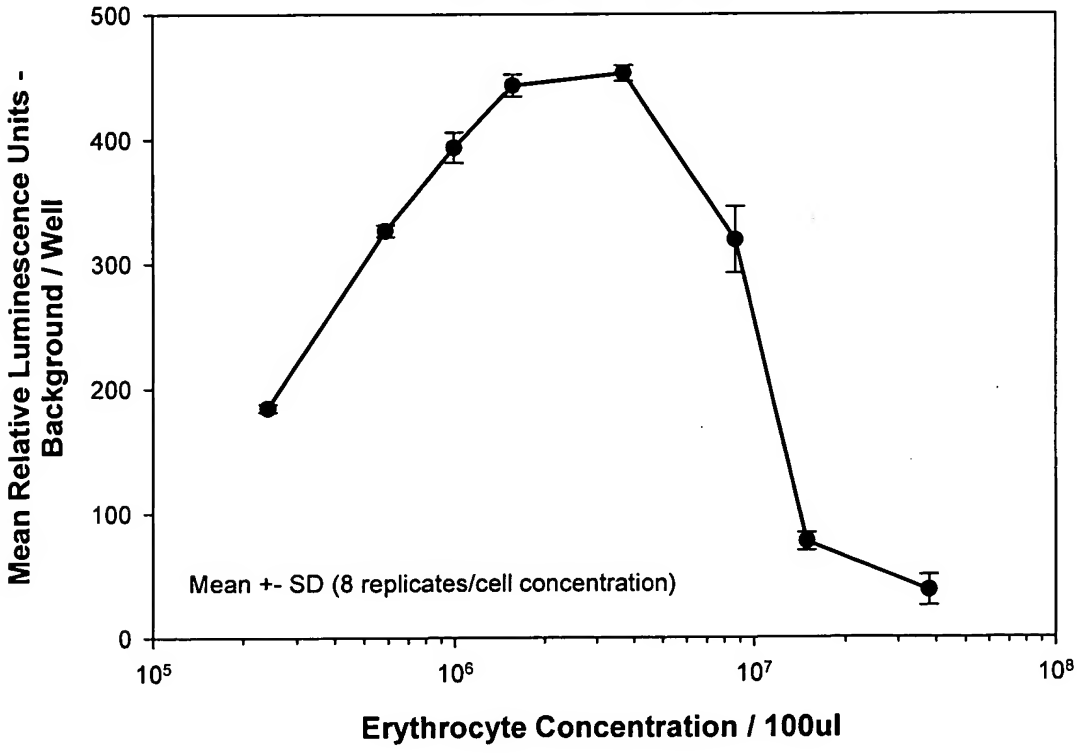


Fig. 2

The Effect of Normal Peripheral Blood Erythrocytes on Luminescence



DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Under 37 CFR § 1.63; includes reference to PCT International Applications)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention ENTITLED:

High-Throughput Stem Cell Assay Of Hematopoietic Stem And Progenitor Cell Proliferation

the specification of which:

- ☐ is attached hereto
- ☒ was filed on January 29, 2002 as:
- ☒ United States Application Serial No. 10/059,521
- ☐ as a National Phase or Continuation or Continuation-in-Part or Divisional of PCT Application No. _____, filed _____ and designating the U.S., and published as _____ on _____
- ☐ with amendments through _____ (if applicable, give details).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT International application(s) designating at least one country other than the United State of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT International applications designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

Country (or PCT)	Application Number:	Filed (Day/Month/Year)	Priority Claimed: Yes	No
			☐	☐
			☐	☐

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States application listed below:

60/264,796	January 29, 2001
(Application Number)	(Filing Date)

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. (or U.S.-designating PCT) Application(s) [list additional applications on separate page]:

U.S. Serial No.: Filed (Day/Month/Year) PCT Application No. Status (patented, pending, abandoned)

I hereby appoint Thomas J. Kowalski, Registration No. 32,147, and David J. Hayzer, Registration No. 43,329 and Frommer Lawrence & Haug LLP, or their duly appointed associate, my attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therewith, and to insert the Serial Number of the application in the space provided above, and specify that all communications about the application are to be directed to the following correspondence address:

Thomas J. Kowalski, Esq.
c/o FROMMER LAWRENCE & HAUG LLP
745 Fifth Avenue
New York, NY 10151

Direct all telephone calls to: (212) 588-0800
to the attention of: Thomas J. Kowalski

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

INVENTOR(S):

Signature: Ivan N. Rich Date: 5/13/05

Full name of sole or first inventor: Ivan N. Rich
Residence: 530 Paisley Drive, Colorado Springs, CO 80906
Citizenship: United Kingdom

Gene Therapy (2000) 7, 1333–1336

www.nature.com/gt

NONVIRAL TRANSFER TECHNOLOGY

TECHNIQUES

Haemoglobin interferes with the ex vivo luciferase luminescence assay: consequence for detection of luciferase reporter gene expression in vivo

M Colin[1], S Moritz[1], H Schneider[2,3], J Capeau[1], C Coutelle[2] and MC Brahimi-Horn[1]

[1]*INSERM U402, Faculté de Médecine Saint-Antoine, Paris, France; and* [2]*Section of Molecular Genetics, Division of Biomedical Sciences, Imperial College, School of Medicine, Sir Alexander Fleming Building, London, UK*

The luciferase reporter gene is a useful tool for determining the efficacy of transfection of plasmid DNA and adenovirus-mediated gene transfer in vivo. However, we report here that the haemoglobin present in tissue samples can mask the detection of the luciferase activity and lead to underestimation of the luciferase levels. We evaluated the degree of interference in different organ samples of mice and investigated the possibilities for removal of haemoglobin from tissue samples by: (1) perfusion of the whole animal; (2) different hypotonic treatments lysing preferentially red blood cells; and (3) chromatographic separation. Removal of haemoglobin resulted in significantly improved detection of luciferase activity from tissue samples. The results indicate that the luciferase activity determined in tissue samples may not reflect the actual level of reporter gene expression, if contaminating blood is not taken into consideration. Gene Therapy (2000) 7, 1333–1336.

Keywords: *adenovirus; gene therapy; haemoglobin; luciferase; perfusion; red blood cells*

Luminescent reporter gene assays for luciferase are frequently employed to determine the efficiency of gene transfer *in vitro*, as they allow quantitative evaluation of luciferase gene expression after transfection. Numerous studies *in vivo* have used this assay to analyse the gene transfer efficacy of plasmid DNA by different liposome preparations *in vivo*[1–7] and some investigations have also examined the efficacy of transduction by adenoviral vectors carrying the luciferase gene after administration to mice by different routes.[8,9]

After performing successful nonviral integrin-mediated gene transfer by an RGD-oligolysine vector using a luciferase reporter gene *in vitro*[10–12] we started testing this vector system *in vivo* in mice. The extremely low levels of luciferase activity in these experiments and similar reports in the literature[1–6] led us to question the sensitivity of detection of luciferase activity by the assay generally employed for animal tissues and to investigate possible factors that may interfere with the luciferase assay, in particular whole blood.

In initial model experiments we added different amounts of whole mouse blood, haemoglobin or plasma to a constant amount of lysed tracheal cells expressing a luciferase reporter gene, and evaluated the luciferase activity by luminometry. Both whole blood and haemoglobin, the latter at a concentration approximately equivalent to that of whole mouse blood led to a similar decrease in the detected luminescence (Figure 1). Neither plasma (blood collected in the presence of EDTA thus containing coagulation factors) (Figure 1) nor serum (clotted blood devoid of coagulation factors) (data not shown), added in the same volumes as whole blood, resulted in a significant decrease in the relative light units (RLU). Addition of lysate buffer instead of blood or haemoglobin did not modify the control value (data not shown). These results indicate that it is the haemoglobin in the blood which interferes with the assay and suggest that haemoglobin contamination results in a considerable decrease in the detectable luciferase activity.

Theoretically it may be possible to calculate the degree of interference if the haemoglobin content of the different organs is known. We therefore estimated the haemoglobin content of organ samples by colorimetry using Drabkin's reagent. The degree of interference could then be determined based on the data shown in Figure 1. It was highest for the lung and slightly lower for both the liver and the spleen (Figure 2). Chloroform extraction before analysis was required to remove lipid which interfered with the colorimetric estimations. During this procedure some loss of haemoglobin occurred, which may have resulted in a slight underestimation of the amount of haemoglobin.

Having established that haemoglobin interferes with the assay, we hypothesised that dilution of the samples may reveal a greater amount of luciferase activity. Indeed, a dilution factor of 10 for the liver and spleen homogenates led to a seven- and 2.8-fold increase in detectable luciferase activity, respectively, and a dilution factor of four for the lung led to a 1.7-fold increase (Figure 3).

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Received 7 January 2000; accepted 3 May 2000

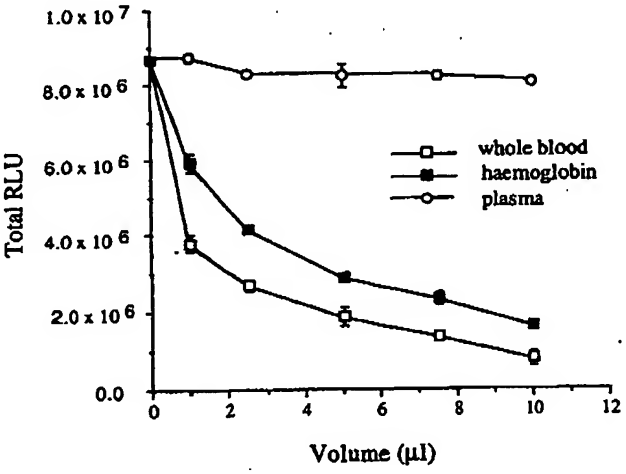

Figure 1 Blood components interfere with the luciferase assay. Different amounts of whole mouse blood (□), haemoglobin (140 mg/ml) (■) or plasma (○) were added to a constant amount of cell lysate (50 µl) following transfection with a luciferase reporter gene plasmid and the relative light units (RLU) were determined using a luminometer. Fetal human tracheal epithelial cells, 56FHTE8σ⁻, were transfected with a vector complex composed of the American firefly (Phoptinus pyralis) luciferase gene plasmid pGL3 (Promega, Charbonnières, France), a $[K]_{16}$RGD peptide and the cationic lipid lipofectamine at a ratio of 1:5:18 (w:w:w), respectively. Cells were harvested in lysis buffer (25 mM Tris/H_3PO_4, pH 7.8, 1% Triton X-100, 15% glycerol, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT) for 15 min at 4°C. The cell lysate was then transferred to 1.5 ml Eppendorf tubes and centrifuged for 10 min at 21700 g. Luciferase activity of the cell lysate was then determined on aliquots (100 µl) using a Berthold luminometer (Lumat LB9507, Evry, France) with a 10 s integration period after automatic injection of 100 µl of a D-luciferin solution (lysis buffer without DTT, with 100 mM ATP, 43 mg/ml D-luciferin). The spectral sensitivity of the photomultiplier covers a range between 390 and 620 nm. Results are representative of three individual experiments each performed in triplicate.

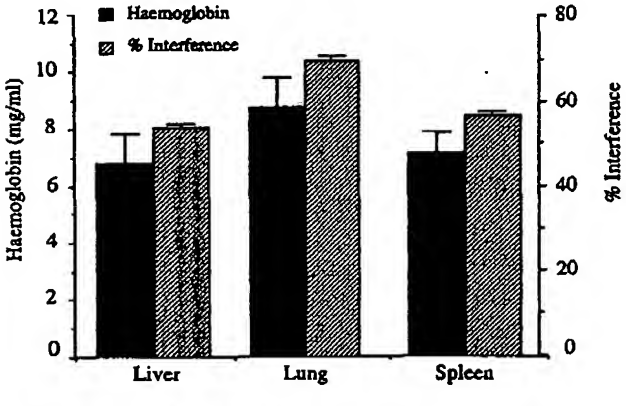

Figure 2 Percentage of interference with the luciferase assay by haemoglobin in homogenates of mouse organs. The theoretically possible interference was calculated from the data in Figure 1 and from the estimation of the haemoglobin content estimated colorimetrically using Drabkin's reagent of a kit for total haemoglobin quantification (Sigma Aldrich, St Quentin Fallavier, France). The percentage decrease in RLU/mg was plotted against the amount of haemoglobin and the percentage interference determined knowing the haemoglobin content of the different samples. Liver, lung and spleen were homogenised in lysis buffer (as above) at 4°C and centrifuged for 10 min at 21700 g. The supernatant was recovered, lipid extraction with chloroform:sample (1:1, v:v) performed and an aliquot used for haemoglobin estimation.

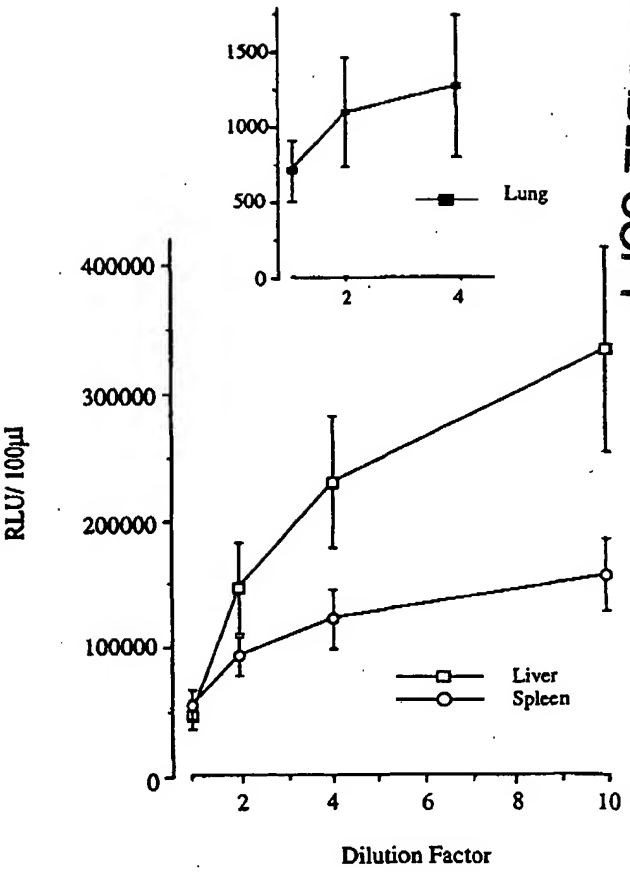

Figure 3 Dilution of organ homogenates results in an increase in the detectable luciferase activity. Mice were injected i.v. (tail vein) with 10^8 $decp_{50}$ of a type 5 adenovirus obtained from Trangene SA, Strasbourg, France, carrying a luciferase gene (AdMLPLuc) under the major late promoter and killed after 72 h with a lethal dose of ketamine. Liver, spleen and lungs were removed, rinsed in PBS, diced and half of each organ was homogenised in 1 ml lysis buffer. The luciferase activities of aliquots (100 µl) of liver, spleen and lung homogenates undiluted or diluted by a factor of 2, 4 and 10 were determined as described in Figure 1. Results are the mean ± s.e.m. for homogenates from five mice. Ketamine at concentrations estimated to be present in organs of the mice does not affect the luciferase activity (data not shown).

Although the emission spectrum of firefly luciferase peaks at 562 nm (SWISS-PROT entry LUCI PHOPY), it ranges from 510 to 650.[13] The major peak of haemoglobin light absorption occures at 408 nm, as indicated above for haemoglobin quantification. However, haemoglobin exists in several different forms (O_2Hb, COHb, HHb and MetHb) each of which has different absorbance spectra. We examined the light absorbance spectra of the different mice organ homogenates, haemoglobin and mice whole blood to determine the degree of overlap with the emission spectrum of luciferase (Figure 4). Substantial absorbance at the peak values of 575 and 540 nm was observed in diluted homogenates. Thus these forms of haemoglobin are probably responsible for the interference since they overlap with the luciferase spectrum.

Next therefore, we sought to remove haemoglobin from the tissue samples before determination of the luciferase activity. Three different approaches were investigated: (1) transcardiac perfusion of mice with an isotonic buffer; (2) preferential lysis of red blood cells

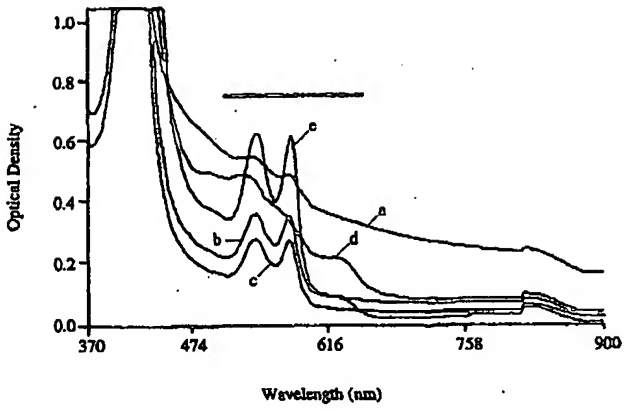

Figure 4 Visible absorption spectra of mouse organ homogenates. The spectra of liver (a) (1/375 dilution), lung (b) (1/125 dilution) and spleen (c) (1/125 dilution) homogenates and of mouse haemoglobin (d) (1.4 mg/ml) and mouse whole blood (e) (1/75 dilution) are presented. The bar represents the range of the luciferase emission spectrum as described previously.[13]

with hypotonic buffers; and (3) chromatographic separation of the luciferase protein from haemoglobin in homogenates.

Transcardiac perfusion was performed through the left and right ventricles and resulted in visible removal of blood from the liver and lungs. This is reflected in the decrease in the estimated haemoglobin content of these organs (Figure 5a and b). Measurement of the optical density at 408 nm, the optimum wavelength for haemoglobin light absorption, is an easy and rapid method for determining the efficacity of the perfusion. The lung was the most efficiently perfused organ. This procedure also resulted in a substantial increase in the detectable luciferase activities, in particular for the liver (six-fold) and the lung (eight-fold) (Figure 5c) while the spleen showed a lower increase (two-fold).

We then investigated two different hypotonic treatments allowing preferential lysis of red blood cells for removal of haemoglobin. Such techniques are used to remove erythrocytes from lymphocyte and spleen cell preparations.[14]

To achieve this, tissues of mice injected with AdMLPLuc were diced very finely and incubated on ice for a short period in either: (1) PBS; or (2) a hypotonic Tris-HCl buffer containing either 5 mM $MgCl_2$ and 10 mM NaCl or 144 mM NH_4Cl. The supernatant containing haemoglobin was removed and the remaining pellet was washed twice before lysis and assay for luciferase activity. This hypotonic treatment resulted in a slight increase in the detected luciferase activity in the liver (1.3-fold), but also in reduced activity in the spleen and lung samples which may be due to the loss of some luciferase activity during hypotonic treatment.

The separation of the luciferase protein from haemoglobin was also attempted by other techniques including ammonium sulphate precipitation and chromatography. Firefly luciferase has been reported to salt-out at 50 to 60% $(NH_4)_2SO_4$,[13] while haemoglobin precipitates at 85%.[15] However, when an aliquot of tissue homogenates in lysis buffer was brought to 60% $(NH_4)_2SO_4$ the precipitate still contained some haemoglobin, and in the case of the liver it was no longer soluble in lysis buffer. As firefly

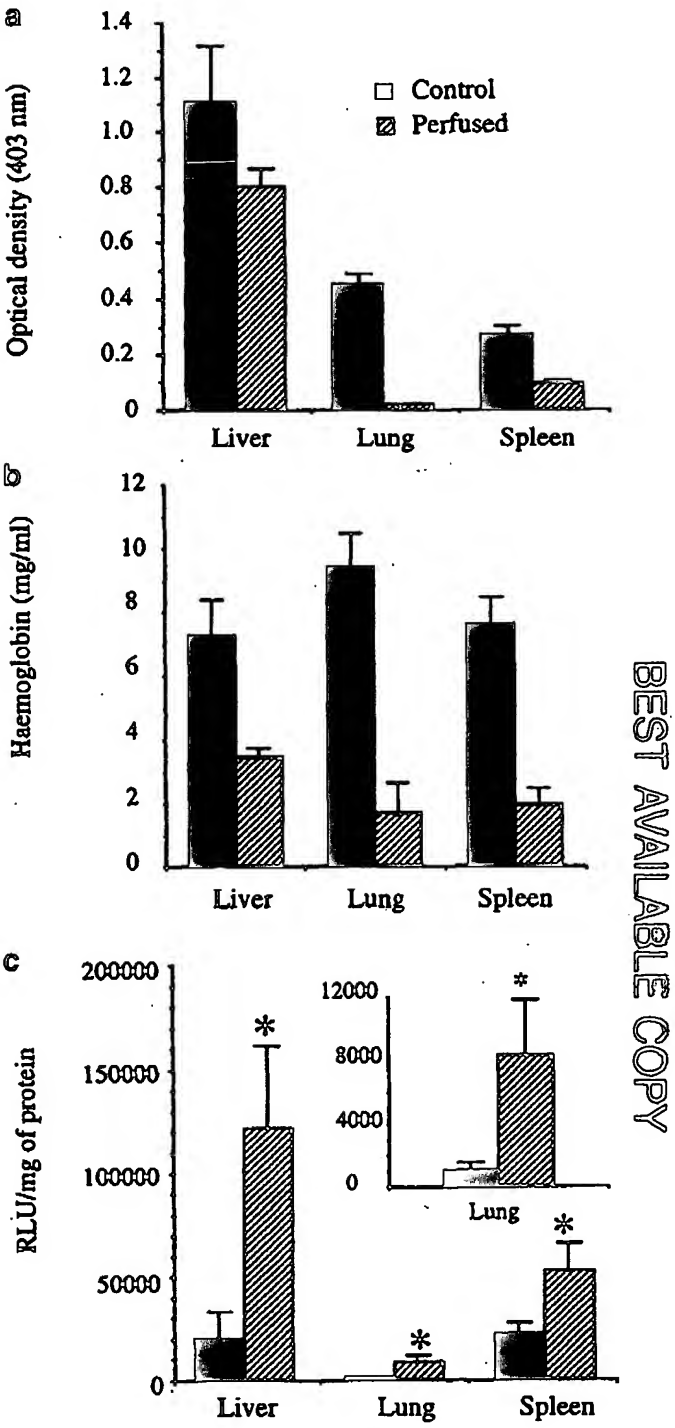

Figure 5 Transcardiac perfusion results in decreased haemoglobin content and increased detectable luciferase activity in mouse organ homogenates. Mice were injected into the tail vein with 100 µl·(5 × 10^{10} p.f.u.) of AdMLPLuc, 48 h later anaesthetised terminally and perfused with 2 × 20 ml of PBS containing 2000 units of heparin/500 ml over a period of about 5 min, first through the left ventricle and then through the right ventricle. Organs were homogenised in lysis buffer. (a) Haemoglobin content estimated by spectroscopy at 408 nm. (b) Haemoglobin content estimated by colorimetry using Drabkin's reagent. (c) Luciferase activity determined as described in Figure 1. Control (full bars), perfused (hatched bars). The protein concentration of the homogenates was determined using the Bradford method.[16] *Results are the mean ± s.e.m. for six mice. Statistical analysis was performed using the nonparametric Mann–Whitney U test. *Probability values <0.05 were considered statistically significant.*

luciferase has a lower pI than haemoglobin ion-exchange chromatography was also tested but, unfortunately, substantial separation was not achieved. In addition, high salt concentrations (200 mM $MgCl_2$) were required to elute the luciferase from the anion exchanger and we noted that the luminescence reaction was sensitive to the salt concentration. Since haemoglobin contains twice as many histidine residues as luciferase we also tested immobilized metal affinity resines usually used for purification of histidine-tagged proteins. However, we were unable to separate the two proteins satisfactorily even by elution with imidazol which competes with histidine for binding. Thus all these alternative techniques investigated appear less efficient than transcardiac perfusion.

The observed decrease in the detectable luciferase activity in the presence of haemoglobin is most likely due to quenching of the luminescence that is emitted by the reaction of luciferase with its substrate D-luciferin, rather than to direct inhibition of the luciferase activity by haemoglobin. This is suggested by the increase in the detectable luciferase activity after dilution of organ homogenates which decreases the absorption at wavelengths corresponding to the emission peak of luciferase (562 nm; yellow–green). However, direct interaction between the two proteins can not be excluded.

There exists several luciferases other than the North American firefly luciferase used in the present study. Each has a different emission maxima: the North American firefly, *Photinus pyralis* (LUCI PHOPY); Japanese firefly, *Luciola cruciata* (LUCI LUCCR); the sea pansy, *Renilia reniformis* (LUCI RENRE); the sea firefly, *Vargula hilgendorfii* (LUCI VARHI) have lambda max at 562, 544, 480 and 460 nm, respectively. Detection of the activity of all these enzymes would be reduced in the presence of haemoglobin. However, mutant Japanese firefly luciferase producing orange (lambda max, 607 nm) and red light (lambda max, 609 and 612 nm) have been reported and thus their emission should not be quenched by haemoglobin,[17] though the detector used would also be required to detect in the red. The spectral sensitivity of the photomultiplier of the luminometer used in this study covers a range between 390 and 620 nm with greatest sensitivity in the blue range. This instrumentation is also the most frequently used by most investigators interested in evaluating gene transfer. In addition, other broad spectrum detectors used in low light detection such as charge coupled devices (CCD) cameras may compensate for some of the effect of hemoglobin on yellow–green luciferase detection.

Nonetheless our results indicate that it is advisable to remove a maximum amount of haemoglobin when performing quantitative analysis of luciferase activity in organs. The perfusion of the whole animal appears to be an effective procedure for removal of haemoglobin from organs. We were particularly interested in the lung and therefore chose to perform intracardiac perfusion. This has allowed us to establish the principle of perfusion as a technique of circumventing the interference of haemogolobin in the luciferase assay. However, perfusion of the liver is probably best performed directly through the portal vein.

In conclusion, our results suggest that luciferase activity levels in previous reports *in vivo* may have been underestimated due to the presence of blood.

Acknowledgements

This work was supported by grants from the 'Institut National de la Santé et de la Recherche Médicale' (INSERM) and from the 'Association Française de Lutte contre la Mucoviscidose'. We thank Dr A Pavirani of Transgene SA (Strasbourg, France) and Dr M Themis (Imperial College, London) for providing AdMLPLuc and Dr D Gruenert (Gene Therapy Core Center, USA) for the 56FHTE8o$^-$ cell line. CC is funded by the Muller Bequest/CF-Trust, the MRC and the March of Dimes Birth Defects Foundation. HS was supported by the Deutsche Forschungsgemeinschaft.

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